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Award Number:
W81XWH-08-1-0631

TITLE:
Analysis of Dachsous2 in Breast Cancer Progression and Recurrence

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REPORT DATE:
October 2010

TYPE OF REPORT:
Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 01-10-2010		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 2 SEP 2009 - 1 SEP 2010	
4. TITLE AND SUBTITLE Analysis of Dachsous2 in Breast Cancer Progression and Recurrence				5a. CONTRACT NUMBER W81XWH-08-1-0631	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Helen McNeill, Email: mcneill@mshri.on.ca				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Mt. Sinai Hospital, 600 University Ave, Toronto, Ontario Canada M5G 1X5				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) US Army MEDical Research and materiel Command Ft. Detrick, MD 21702				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Microarray analysis of tumors women with axillary node negative (ANN) breast cancer revealed that a cDNA for Dachsous 2 (Ds2) is one of the most highly discriminatory genes in tumors from patients who had experienced early recurrence, compared to tumors from patients who did not recur for at least ten years ($p = 1.1 \times 10^{-7}$ by students t-test). This suggests that increased transcription of Ds2 may be a predictive indicator of recurrence in ANN breast cancer. Ds2 encodes a large cell adhesion molecule implicated in planar polarity and cell proliferation. To determine the significance of the increased levels of Ds2 in recurrence we will: 1) Validate gene expression array results and determine if Ds2 proteins levels increase in tumors 2) Determine if PCP or Hippo pathway gene expression is altered upon Ds2 overexpression. 3) Determine the effects of altering Ds2 levels on proliferation and tumor susceptibility in the mouse					
15. SUBJECT TERMS Breast Cancer, Ds2, planar cell polarity, Hippo pathway					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES 12	19a. NAME OF RESPONSIBLE PERSON Helen McNeill
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER (include area code) 416-586-8267

Table of Contents

	<u>Page</u>
Introduction.....	1
Body.....	1
Key Research Accomplishments.....	8
Reportable Outcomes.....	8
Conclusion.....	9
References.....	9
Appendices.....	9

INTRODUCTION:

Microarray analysis of tumors women with axillary node negative (ANN) breast cancer revealed that a cDNA for *Dachsous 2* (*Ds2*) is one of the most highly discriminatory genes in tumors from patients who had experienced early recurrence, compared to tumors from patients who did not recur for at least ten years ($p = 1.1 \times 10^{-5}$ by students t-test).

This suggests that increased transcription of Ds2 may be a predictive indicator of recurrence in ANN breast cancer. *Ds2* encodes a large cell adhesion molecule¹. The *Drosophila* homolog of *Ds2*, *Ds*, functions as a planar cell polarity (PCP) ligand for the large cadherin *Fat*²⁻⁵, and together *Ds* and *Fat* regulate tissue organization through a PCP signaling pathway. Recent data has also linked *Ds* with a newly described growth control pathway, the Hippo kinase pathway⁶⁻⁹. To determine the significance of the increased levels of *Ds2* in recurrence we will: 1) Validate gene expression array results and determine if *Ds2* proteins levels increase in tumors 2) Determine if PCP or Hippo pathway gene expression is altered upon *Ds2* overexpression. 3) Determine the effects of altering *Ds2* levels on proliferation and tumor susceptibility in the mouse

BODY:

Task 1. Determine if *Ds2* protein and transcript levels are increased in tumors from patients that have recurrent breast cancer.

A) Generate anti-*Ds2* antibody. Our first goal was to generate specific antibodies to *Ds2* that could be used in paraffin section analysis of tumour samples from ANN patients. We initially generated 4 antisera to the entire cytoplasmic domain of *Ds2*, immunizing 2

Fig. 1

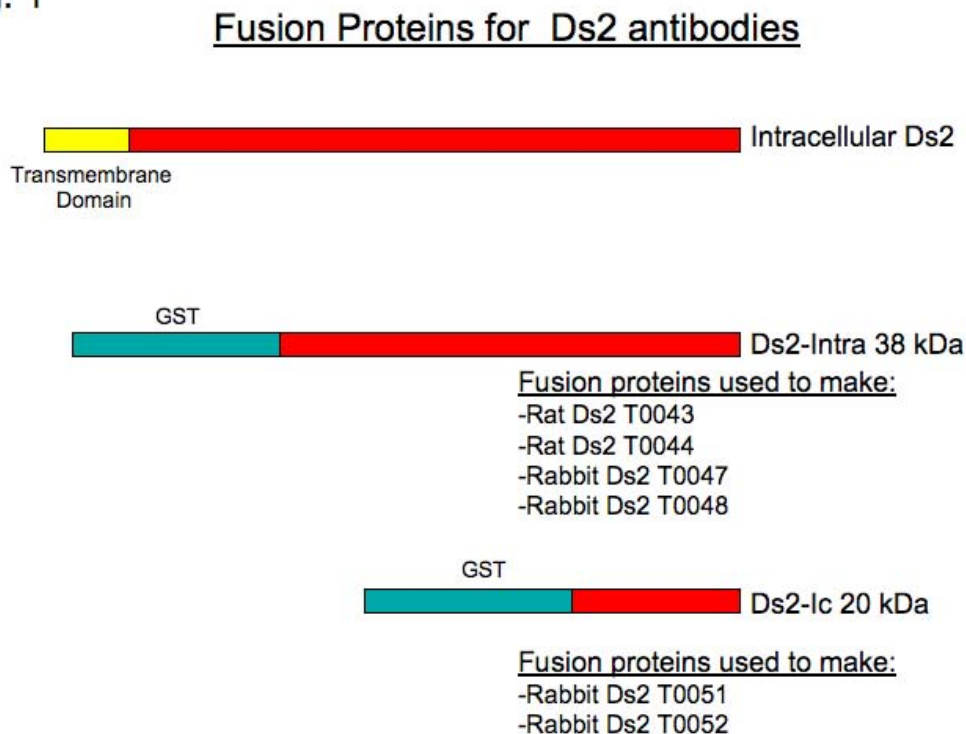


Figure 1. Constructs used to generate fusion proteins for specific antibodies to *Ds2*. Green indicates the GST portion, and red the segment of the intracellular domain of *Ds2* used to generate antibodies.

rats and 2 rabbits (antibodies TO43, 44, 47 and 48: **Figure 1**). We chose to assay the antibodies using an immunohistochemistry of mouse hippocampus, as it has a simple and clear expression pattern of Ds2¹. None of these antibodies showed any clear expression pattern in the hippocampus assay, despite trying a large number of staining conditions (we assayed cryosections and paraffin sections, tried a large dilution series, different blocking and different antigen retrieval approaches) (data not shown). We then generated fusion proteins to smaller portions of the cytoplasmic domain and inoculated 2 rabbits with this antigen (antibodies TO51 and TO52: **Figure 1**). These antibodies showed high specificity in both cryosection analysis and paraffin analysis of the hippocampus (**Figure 2**) and whole embryo staining (**Figure 3**).

Fig. 2 Hippocampus Rabbit anti-Ds2 staining

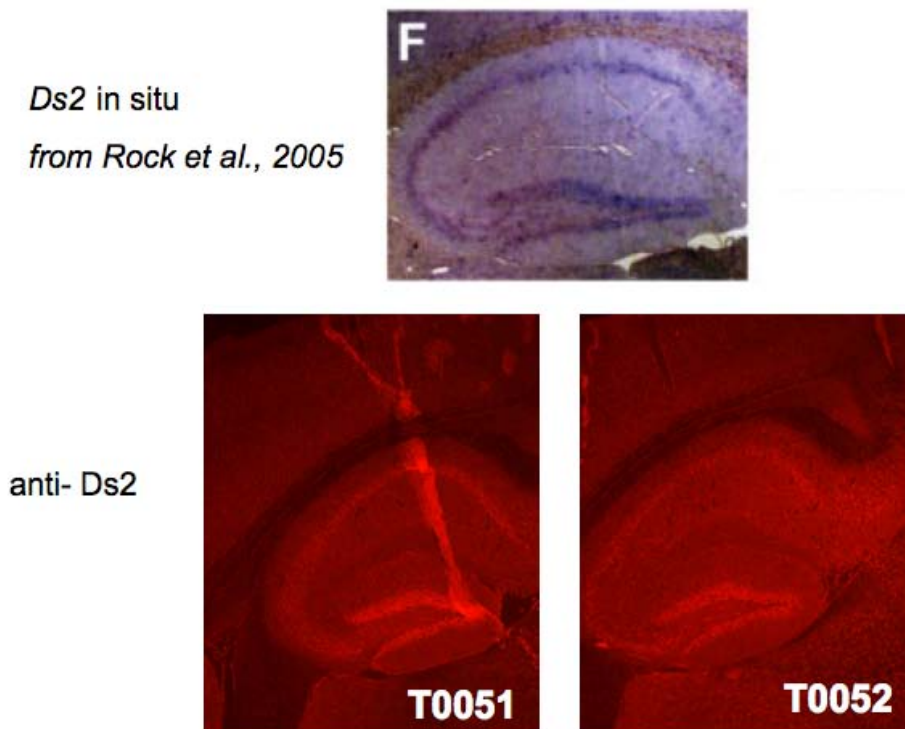


Figure 2. Validation of Ds2 antibodies. Top shows an in situ hybridization expression pattern of Ds2 in the hippocampus (taken from Rock et al., 2005). Bottom shows immunofluorescence of sections of the hippocampus, probes with antibodies to Ds2 (TO51 and TO52) that were effective in detecting Ds2 protein in immunofluorescence and western blot analysis.

Fig. 3

E12.5 embryo anti-Ds2 staining

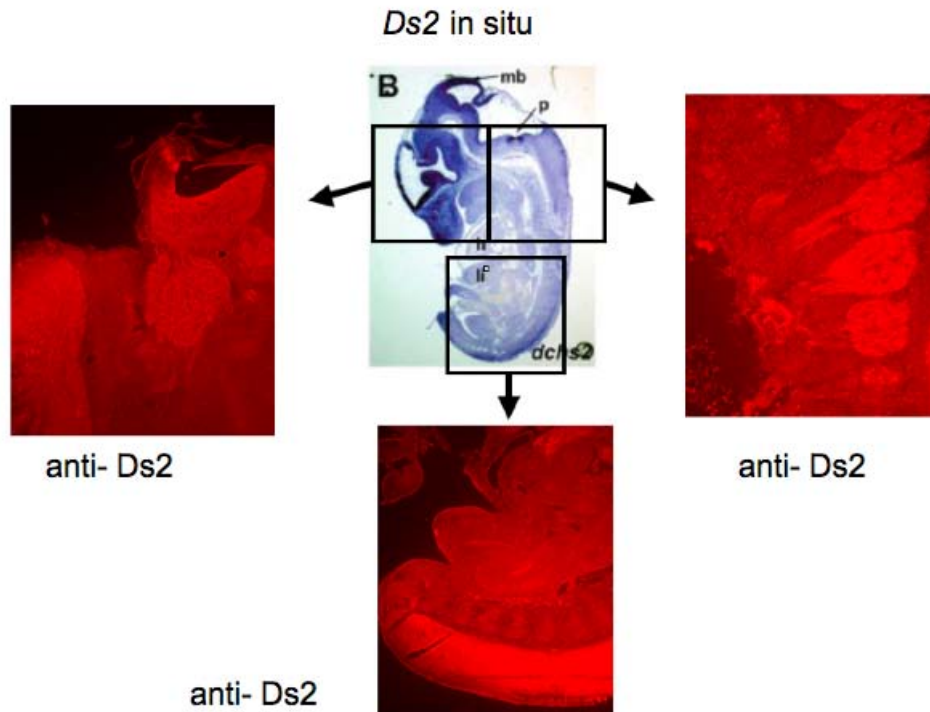


Figure 3. Immunofluorescence using specific Ds2 antibodies reveals the same pattern as published images of Ds2 by in situ hybridization (taken from Rock et al., 2005).

B) Test by RT-PCR if increased Ds2 expression validates in tumour samples. To determine if Ds2 mRNA is increased in tumour samples, we have used RT-PCR in collaboration with the laboratory of Dr. Irene Andrulis. Our initial studies assayed cell lines to optimize PCR conditions. Expression was normalized to the housekeeping gene, *hypoxanthine phosphoribosyltransferase 1* (HPRT1). We found that most cell lines express little or no Ds2 mRNA, although there was detectable expression in some cells such as SKOV3. The highest expression detected in cell lines was found in NTERA-2 cells (**Figure 4** and data not shown). Preliminary analysis of a panel of tumor samples revealed that most had low levels, on the order of most cell lines, however a few have very high levels, even higher than in NTERA-2 cells (**Figure 5**). This suggests that some recurrent tumors may indeed express unusually high levels of Ds2, however the finding thus far are not statistically significant, and more tumours must be assayed to reach a definitive conclusion.

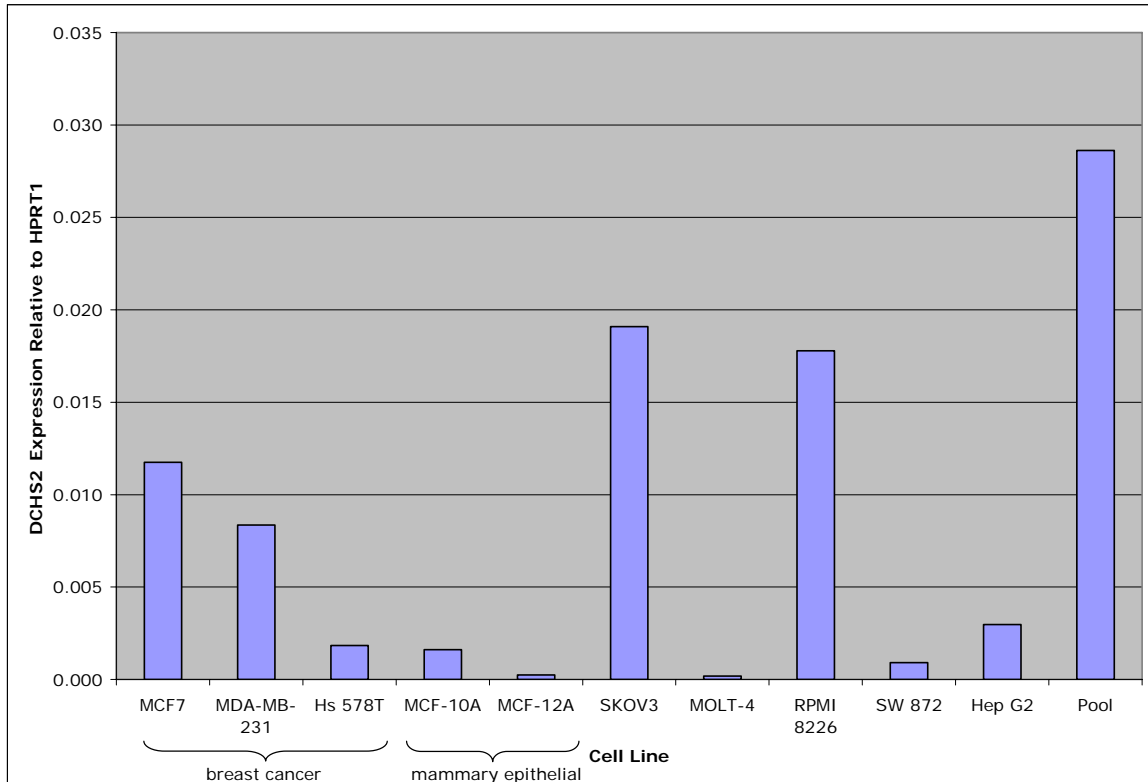


Figure 4. Quantifying expression of *dachsous 2* (DCHS2) in cell lines and a pool of 13 cell lines, using the real-time reverse-transcription polymerase chain reaction. Expression was normalized to the housekeeping gene HPRT1.

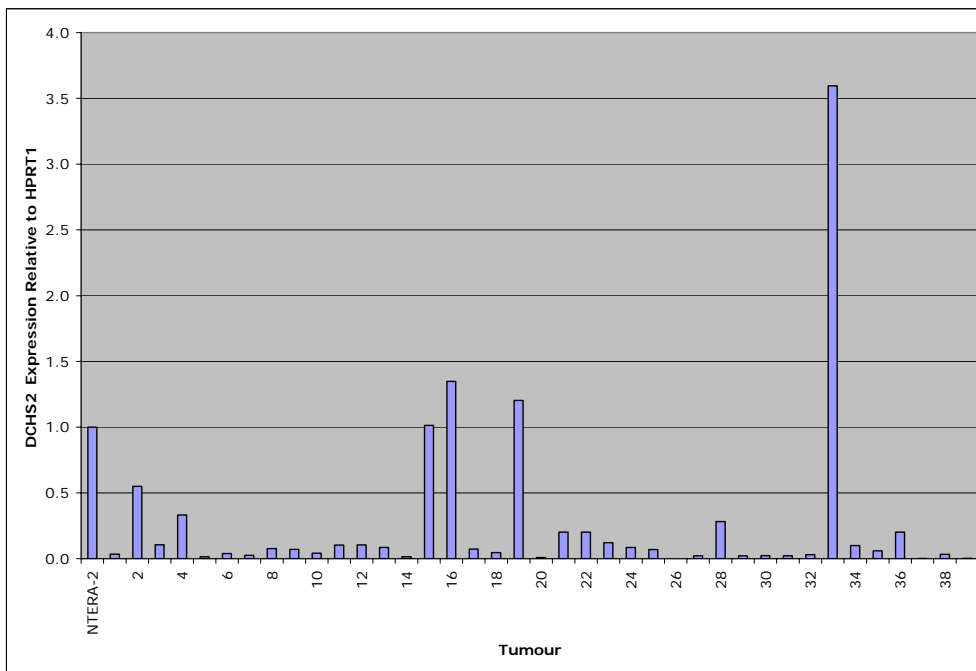


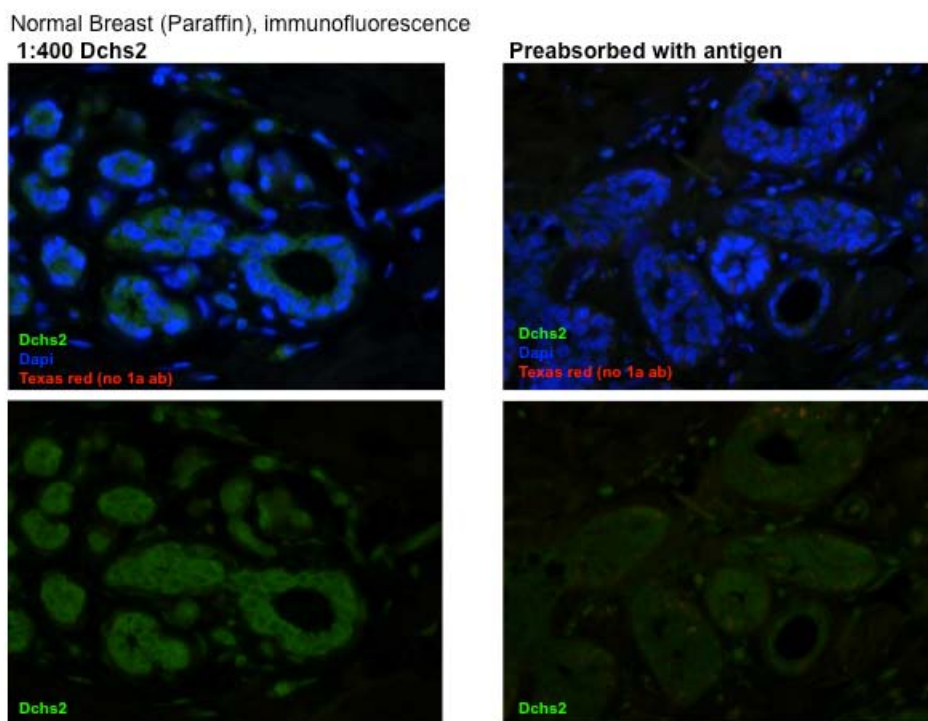
Figure 5. Analysis of Ds2 expression in tumours from ANN patients. Expression was low in most tumours but was very high in a subset of recurrent tumors.

Task 2. Determine if the Hippo or PCP pathway is activated with increased Dachs levels.

1.A) Generate or obtain antibodies against Yap, MST1/2 and generate *in situ* probes for *fjx1*. B) Optimize staining on tissue sections. We have obtained antibodies to YAP and MST1/2 from commercial sources, and have generated *in situ* probes for *fjx1*. We have optimized staining for these reagents.

2A) Optimize TMA staining for Ds2. We have extensively now tested our Ds2 antibodies on normal breast samples and TMAs, with both positive and negative controls(**Figure 6 and Figure 7**). Preabsorbing with Dachsous antigen blocks staining, confirming the specificity of our antibodies on the TMA analysis.

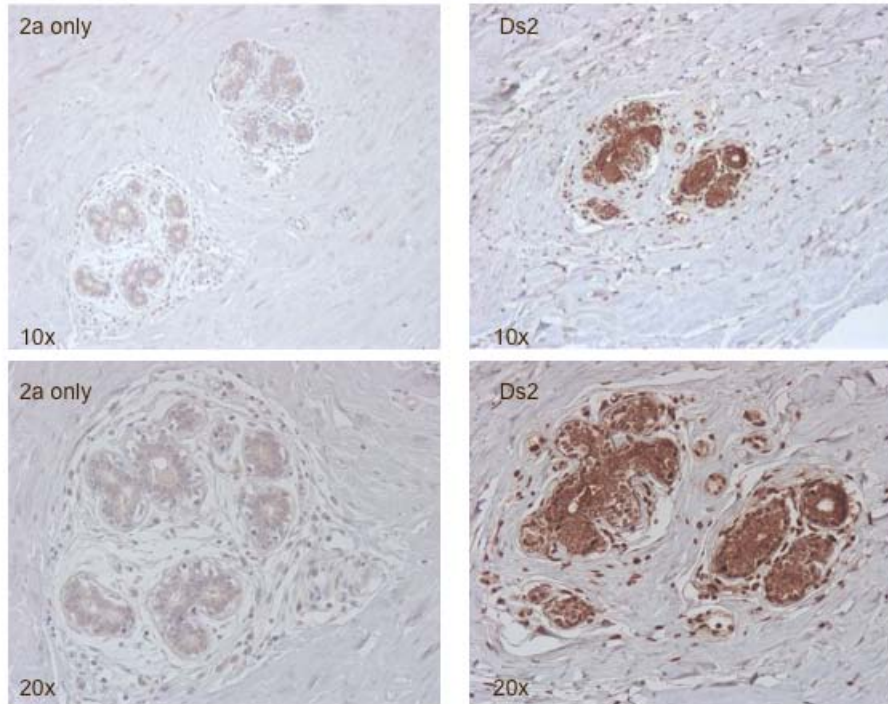
Figure 6. TMA Dchs2 Antibody Testing: Normal Breast



We find that Ds2 is expressed at cell-cell junctions in normal breast tissue, and find that in many breast tumours Ds2 becomes increased and expression and is often mislocalized. Our preliminary analysis of TMAs suggests high levels of Ds2 staining in many tumours, however these TMAs must be quantified and repeated, as the level of staining was higher than in the initial analysis.

Figure 7. TMA Dchs2 Antibody Optimization with DAB

Normal Breast:

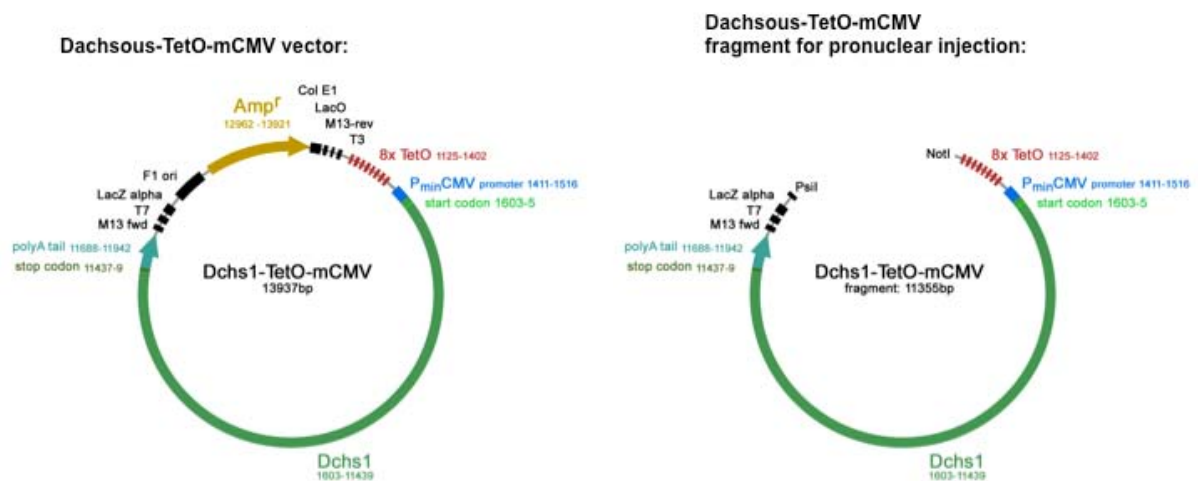


Task 3. Ascertain if overexpression of Ds2 affects tumour incidence or metastasis in mouse models of breast cancer.

A) Generate construct for transgenic analysis of Ds2 function. No functional studies have yet been conducted on Ds2 in mammals, and a full-length Ds2 cDNA was not available. We initiated our cloning of Ds2 cDNA by conducting PCR from embryonic mouse cDNA libraries. We designed our PCR primers based on the available reference sequence predicted from the mouse genome. We generated embryonic cDNA libraries from E17.5 embryos. The full length Ds2 cDNA was predicted to be 8 kb, and we amplified the cDNA in several pieces, as we were unable to amplify the predicted full-length transcript. Sequencing of this transcript revealed several portions that were missing, in comparison to the predicted sequence. Examination of the mouse and human genome indicated that the missing sequences corresponded to predicted Ds2 exons. This indicated that there was unsuspected complexity in the splicing of this transcript. In collaboration with Dr. Lisa Goodrich, we have isolated a number of independent Ds2 transcripts. This raises the question of which is the appropriate Ds2 cDNA to use for transgenic overexpression to assay the effects on the PCP and Hippo pathway in mice. All of the transcripts contain exon 25, which was on the microarray that detected increased *Ds2* in tumours from woman with recurrent breast cancer, therefore this cannot be used to discriminate among these alternate splice forms. To ensure that we used a functional splice form, tested these cDNA by transient transfection into cells that express Fat4. Functional Ds2 should be recruited to cell-cell junctions, upon interaction with Fat4. Generation of

the predicted isoforms of Ds2 did not yield a functional protein. Examination of the extracellular domain of Ds1 and Ds2 isoforms indicated that Ds1 would function to activate the Hippo pathway as well as Ds2. Last year a fully functional Ds1 cDNA was published by Dr. Tanoue's group in Japan, and shown to bind Fat4, the receptor for the pathway. We therefore obtained the cDNA from Dr. Tanoue, and cloned it into a tetracycline-inducible vector (**Figure 8**).

Figure 8 Cloning for a Dachous Tet-ON inducible transgenic mouse



pA-TetO-mCMV vector:

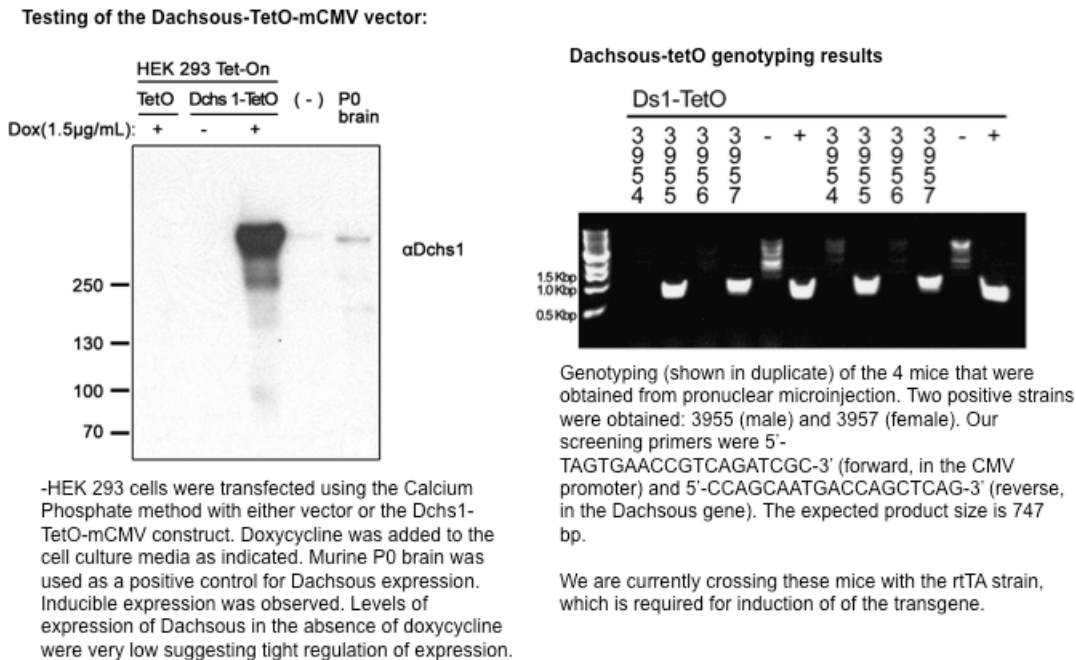
-used for creation of a doxycycline inducible transgenic mouse with ubiquitous dachsous1 expression.

Cloning:

- The vector had a poly(A) tail located after the MCS. Dachous (Dchs) cDNA was cloned into the MCS. The resultant TetO-dachsous-mCMV was digested with restriction enzymes and purified to remove bacterial sequences.
- The transgene was microinjected into pronuclei of the mouse zygotes (pronuclear microinjection) and implanted into pseudopregnant recipients. Pups were screened for the presence of a transgene at 2-3 weeks of age. Transgenic founders were identified by a PCR screening to identify mice that have the Dachous-TetO sequence.
- embryos that were used were the result of the cross of C57BL/6 females with B6D2F1 (C57BL/6 x DBA/2) males.

To determine if the vector was suitable for inducible expression of Dachous, we tested with transient transfection in HEK293 cells that express the rtTA. These studies indicated that this vector conferred tight regulation (**Figure 9**). We generated transgenic mice with this vector through pronuclear microinjection, and obtained two founder lines. We will shortly be testing these mice for in vivo response to Doxycycline.

Figure 9. Generating a Dachsous Tet-ON inducible transgenic mouse



KEY RESEARCH ACCOMPLISHMENTS:

- Determination of novel splice forms of Dachsous
- Generation and validation of antibodies specific to Dachsous
- Initial screening of TMAs for Dachous expression
- Generating mice transgenic for Doxycycline-inducible expression of Dachsous
- Identification of high levels of Ds2 in a subset of tumors.

REPORTABLE OUTCOMES

-Specific Antibodies to Ds2

-transgenic mice expressing a Doxycycline-inducible Dachsous construct

CONCLUSION:

In previous studies we identified increased levels of *Dachsous* mRNA as a predictor of recurrence in woman with axillary node negative breast cancer. This lead to the hypothesis that increased levels of *Dachsous* mRNA and protein may provide a biomarker for recurrent breast cancer. To test this hypothesis, we have generated specific antibodies to Dachsous, optimized Dachsous staining on paraffin sections, cloned full length Dachous cDNAs and obtained antibodies and in situ probes to components of the mammalian Hippo and Planar Cell Polarity Pathway . We have also generated mice that

have the potential to overexpress Dachshous upon treatment with Doxycycline. These tools will allow us to test if increases in Dachshous is a good biomarker for recurrent breast cancer. In addition we will use these tools to determine the signal transduction pathways that are disrupted when Dachshous is overexpressed. These tools will allow us to determine if overexpression of Dachshous is a causal factor in breast cancer recurrence.

APPENDICES: N/A

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